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A STUDY OF AMMONIA PRODUCTION BY A CERTAIN STRAIN OF AVIRULENT HUMAN TUBERCLE BACILLUS

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Kendall, Day and Walker[1] say: "A study of the nitrogenous metabolism of certain rapidly growing acid-fast bacteria, so-called saprophytic human tubercle bacilli, in plain glucose, mannitol and glycerol broths, revealed the fact that the organisms produced moderate amounts of ammonia. . . . The maximum production of ammonia was reached about the third week in plain glucose and mannitol cultures, the reaction becoming progressively alkaline during this period. In glycerol medium, on the other hand, the increase in ammonia was progressive to the fourth week, although the rate of production was slower and the reaction became acid, contrasting sharply in this respect with the alkalinity of the other cultures. The ammonia curve gradually diminished from the maximum, particularly in plain, glucose and mannitol mediums, until scarcely half the amount remained in solution at the end of the sixth week. . . . A definite explanation for this phenomenon of waxing and waning of ammonia production is not apparent, but available evidence points to a coincidence between luxuriance of growth and ammonia increase followed by autolysis of the organisms and a decrease in ammonia." They go on to state that "total nitrogen determinations of the bacteria-free cultures show a small but unmistakable decrease at the height of bacterial development, indicating the incorporation of this element in the bacterial bodies, followed later by an increase in nitrogen, suggesting a gradual autolysis of the organisms with the liberation of some of the nitrogen in soluble form."

In this connection Kendall, Day and Walker apparently have not determined the total nitrogen of the bacterial bodies themselves (merely of the clear underlying broth) to see whether there actually was an increase in the nitrogen content of the bacteria which would be necessary to prove their statement.

In our first experiment the organism was inoculated into a sufficient number of Erlenmeyer flasks, each containing 100 c c of 1% dextrose broth, so that duplicate flasks could be studied at weekly intervals for a period of 6 weeks. In this and the following experiments an avirulent culture of the tubercle bacillus obtained from the American Museum of Natural History, New York, designated as "597" was

Received for publication Oct. 28, 1921.

[1] Jour. Infect. Dis., 1920, 26, p. 45.

used. The analysis comprised the determination of a titratable reaction using phenolphthalein as an indicator, the measure of ammonia by Folin's method, the determination of the total nitrogen content of the medium plus the bacterial growth in contrast to Kendall's method of determining only the total nitrogen in the bacterial free broth.[2]

The data obtained are given in table 1, and bring out the following points: The medium, which is acid toward phenolphthalein at the beginning of the experiment, gradually undergoes a change in reaction until it becomes quite strongly alkaline toward phenolphthalein. After the medium becomes alkaline the ammonia and the total nitrogen contents both decrease. The steady gradual decrease in the amount of nitrogen in the total contents of the flasks shows that the loss of nitrogen noted by Kendall, Day and Walker, cannot be wholly attributed to the incorporation of nitrogen within the bacilli.

TABLE 1

Metabolism of Tubercle Bacillus

Age in Days	Dextrose Broth			Glycerol Broth		
	Reaction Toward Phenol-phthalein	Total Nitrogen	Nitrogen Present as Ammonia	Reaction Toward Phenol-phthalein	Total Nitrogen	Nitrogen Present as Ammonia
Control	+1.8*	0.3346	......	+1.00	0.3892	
7	+0.8	0.3206	0.0300	+0.9	0.3547	0.0243
14	−2.0	0.2905	0.0435	+1.7	0.3554	0.0238
21	−3.0	0.2748	0.0120	+0.5	0.3561	0.0305
28	−2.3	0.2619	0.0020	+0.6	0.3511	0.0282
35	.....	0.2459	0.0025	No reading made	0.3512	0.0252
42	.....	0.2213	......	No reading made	0.3270	0.0242

* Figures represent the acidity (+) or alkalinity (—) of 100 c c of medium expressed as cubic centimeters of normal acid or alkali.

A similar experiment was made with glycerol broth as a medium and these data also are given in table 1. In this case the acid reaction of the medium persisted longer than in the case of the dextrose broth. The total nitrogen content remains constant during this period of acid reaction while the ammonia content increased during the same period. When the amount of total nitrogen began to decrease the ammonia also began to decrease. Unfortunately the inadvertent absence of the reaction data does not permit us to determine in this instance whether the decrease of total nitrogen and loss of ammonia are coincident with

[2] Ibid., 1914, 15, p. 423.

the advent of an alkaline reaction of the medium. Experiments reported in another table in this paper, however, show this to be the case.

From the results obtained with dextrose broth we were led to suspect that the decrease in the nitrogen and ammonia contents was due simply to volatilization of ammonia caused by the alkaline reaction of the medium and the heat of the incubator. If nitrogen were lost in this fashion, the trapping and measuring of the escaping ammonia would prove or disprove this assumption. In order to do this the following experiments were performed:

Four culture mediums were used, plain broth, 1% mannitol broth, 1% dextrose broth and 3% glycerol broth. The plain broth (sugar-free broth made from meat juice) was used as a basis for the other 3. The apparatus for growing the organism and collecting the volatilized ammonia was set up in the following manner: 18 clean and sterile 8 ounce, wide mouthed bottles, each containing 50 c c of culture medium were inoculated with the bacillus; 18 similar bottles, each containing 15 c c of normal hydrochloric acid, 40 c c of distilled water (or sufficient water to cover the end of the glass tube), and 3 drops of alizarin, were paired with the culture bottles and connected by means of glass tubes and rubber stoppers. The 36 bottles, culture and acid bottles alternating, were then connected with each other in an incubator kept at a temperature of 37, one terminal joined to a wash bottle containing diluted sulphuric acid and the other terminal to a suction pump. In this manner a constant stream of ammonia free air passed over the cultures, at the same time drawing any volatilized ammonia from each culture bottle into the succeeding acid trap bottle.

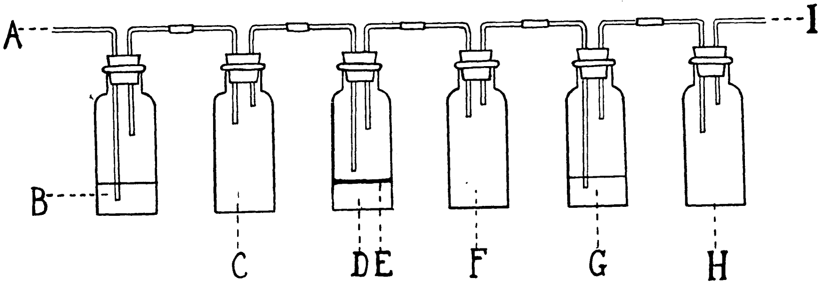

Fig. 1.—A, open to air; B, diluted sulphuric acid (5%); C, empty bottle; D, culture medium; E, pellicle; F, empty bottle to save culture from possible back flow of acid; G, trap bottle containing standardized acid; H, empty trap bottle connected with suction pump at I. (Any number of sets, D, F. G, may be inserted in the series.)

The experiment was set up to cover a period of 6 weeks, 3 pairs of bottles being removed each week. The acid in each trap bottle was titrated with standard sodium hydroxide to determine the amount of volatilized ammonia which had been trapped. The contents of 2 culture bottles were used for total nitrogen determinations in duplicate according to the Kjeldahl method. It may be noted here that the entire contents of the bottles, broth and pellicle, were used. From the third culture bottle were taken two 10 c c portions of the medium for duplicate ammonia determinations according to the Folin air method, and portions for the determination of the reaction of the medium toward phenolphthalein and alizarin. The data collected are summarized in table 2.

TABLE 2

Ammonia Production by Tubercle Bacillus in Plain, Dextrose, Mannitol and Glycerol Broths

Age in Days	Broths	Reaction Toward Alizarin	Reaction Toward Phenolphthalein	1 Grams Nitrogen in Culture Bottle	2 Grams Nitrogen Caught in Trap as Ammonia	3 Total Nitrogen 1 + 2	4 Grams Nitrogen in Medium as Ammonia	5 Total Ammonia Produced by Organism in Terms of Nitrogen 2 + 4
Control	Plain.......	—3.0*	+1.7*	0.1937	0	0.1937		
	Dextrose....	—3.05	+1.2	0.1563		0.1563		
	Mannitol...	—3.0	+1.6	0.2098		0.2098		
	Glycerol....	—4.0	+0.5	0.1836		0.1836		
7	Plain.......	—6.4	—0.75	0.1901	0.0031	0.1932	0.0266	0.0297
	Dextrose...	—5.1	—1.2	0.1602?	0.0065	0.1667?	0.0202	0.0267
	Mannitol...	—4.8	—0.2	0.2062	0.0031	0.2093	0.0185	0.0216
	Glycerol....	—3.9	0.00	0.1752	0.0074	0.1826	0.0021	0.0095
14	Plain.......	—5.5	—1.7	0.1611	0.0338	0.1949	0.0168	0.0506
	Dextrose....	—6.1	—1.1	0.1359	0.0204	0.1563	0.0239	0.0443
	Mannitol...	—6.8	—1.2	0.2091?	0.0023	0.2114?	0.0261	0.0284
	Glycarol....	—5.1	—0.8	0.1830	0.0041	0.1875	0.0056	0.0097
21	Plain.......	—7.8	—1.2	0.1539	0.0402	0.1941	0.0360	0.0762
	Dextrose....	—6.2	—1.35	0.1325	0.0234	0.1559	0.0221	0.0455
	Mannitol...	—8.8	—1.0	0.2005	0.0094	0.2099	0.0396	0.0490
	Glycerol....	—4.4	—0.75	0.1834	0.0031	0.1865	0.0084	0.0115
28	Plain.......	—6.2	—1.1	0.1367	0.0567	0.1934	0.0208	0.0775
	Dextrose....	—4.6	—1.2	0.1121	0.0439	0.1560	0.0029	0.0468
	Mannitol...	—6.35	—0.95	0.1644	0.0463	0.2107	0.0136	0.0599
	Glycerol...	.....	.....	......	......	......	......	......
35	Plain.......	—7.7	—0.2	0.1329	0.0607	0.1936	0.0229	0.0836
	Dextrose....	—3.9	—0.9	0.1111	0.0460	0.1570	0.0035	0.0495
	Mannitol...	—4.45	—1.05	0.1386	0.0743	0.2129	0.0010	0.0753
	Glycerol....	—4.9	—1.2	0.1737	0.0135	0.1872	0.0155	0.0290
42	Plain.......	—5.0	—1.0	......	0.0744	......	0.0044	0.0826
	Dextrose....	—4.3	—0.8	0.1118	0.0440	0.1558	0.0028	0.0468
	Mannitol..	.....	.....	......	......	......	......	......
	Glycerol....	—4.6	—0.4	......	0.0409	......	0.0242	0.0651

* Figures represent the acidity (+) or alkalinity (—) of 100 c c of medium expressed as cubic centimeters of normal acid or alkali.

Reaction.—The reaction of the medium to phenolphthalein is variably alkaline in every case, though the mediums were at first acid. The alkalinity of the glycerol broth during the activity of the bacilli is at variance with the results obtained by Kendall[2] for saprophytic human tubercle bacilli and the Theobald Smith[3] reaction, according to which human tubercle bacilli cultures become pronouncedly acid in glycerol broth. It also differs from the results obtained in our first experiment (table 1).

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[3] Am. Jour. Med. Sc., 1904, 128, p. 216.

Total Nitrogen.—Our suspicion in regard to the evaporation of ammonia seems to have been justified. A study of the accompanying table shows that in every case, with the exception of the glycerol broth, there is a decrease, from week to week, in total nitrogen content of the bottles (clear broth and pellicle). Accompanying the loss of nitrogen from the bottles there is a compensating increase of nitrogen (in the form of ammonia) in the acid of the trap bottles. This simple statement scarcely needs any elaboration. It is most clearly shown in the experiment with plain broth as a medium, but it is just as evident in the other cases.

Ammonia Production.—If we consider the production of ammonia in the culture medium alone, we find, except in the case of glycerol broth, the "waxing and waning" phenomenon noted by Kendall. However, the total ammonia actually produced by the organism must be sought for both in the culture medium and in the acid of the trap bottle. The sum of these two ammonias will reveal the true ammonia production of the tubercle bacillus. We have expressed it in terms of grams of nitrogen. In no case do we find a fall in the ammonia curve. The maximum amount is produced in plain broth, with mannitol broth a close second; the least is found in dextrose broth. While there is a steady increase in total ammonia in plain broth, mannitol broth and glycerol broth, in dextrose broth there seems to be little increase after the fifteenth day. In glycerol broth the early production of ammonia is very slight.

SUMMARY

A study of the nitrogenous metabolism, during six weeks, of a certain avirulent culture of human tubercle bacilli revealed the fact that there is a continuous production of ammonia in plain, dextrose, mannitol and glycerol broths.

Over 30% of the nitrogen originally present in the medium may be converted to ammonia.

The ammonia produced is partly retained in the medium but the greater part is lost through volatilization.

The loss of ammonia is coincident with an alkaline reaction of the medium.

There was no recession in the production of ammonia by the organisms during the period covered by our experiments (6 weeks).

The "waxing and waning" of ammonia as noticed by Kendall is due to two factors: a continuous production of ammonia by the organism, and a loss of ammonia from the medium into the air by volatilization.

So long as the medium remains acid (see especially the glycerol broth) both the total nitrogen and the ammonia contents of the broth show no decrease.

When the broth becomes alkaline both the total nitrogen and ammonia contents show a decrease.

If ammonia is utilized by the tubercle bacilli the amount is extremely small in comparison with the amount lost by volatilization.